Woods Hole Oceanographic Institution

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WOODS HOLE, MASSACHUSETTS 02543

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FINAL REPORT

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SUMMARY

A study of 24 weeks duration was carried out in which oysters (*Crassostrea gigas*) were grown in four regimes. These were: (i) on phytoplankton cultured in a mixture of secondary treated sewage effluent and seawater for a period of 12 weeks followed by a second 12 week period of feeding on phytoplankton cultured in a "clean," inorganically enriched regime; (ii) as for (i) except that the secondary effluent was sand filtered prior to use; (iii) as for (ii) except that the effluent was charcoal filtered prior to use; and (iv) using "clean," inorganically enriched phytoplankton food for the 24 week duration. At intervals of two weeks, populations of oysters were removed for assay for trace metals (Cd, Cr, Cu, Hg, Ni, Pb, Zn) and organic contaminants (hydrocarbons, P.C.B.'s). No significant accumulation or depuration of any metal or organic contaminant was evident in any of the regimes. In terms of these contaminants all oysters are within acceptable edible standards as set by F.D.A.

A series of experiments was carried out to examine the public health implications of enterovirus survival in a mollusc culture system fertilized with secondary treated sewage effluent. Using MS-2 bacteriophage and vaccine strain poliovirus it would appear that depuration could be effected in 20-25 days in *C. gigas* at 15°C. However this does NOT mean that such a time span would be adequate for other enteroviruses. Further work is required in this area.

INTRODUCTION

Dunstan and Menzel (1971) demonstrated that continuous cultures of marine phytoplankton could be maintained in mixtures of secondary treated sewage effluent and seawater. Subsequently Ryther *et al.* (1972) developed this theme and proposed a waste recycling aquaculture study based on mass cultures of marine phytoplankton, grown in mixtures of secondary sewage effluent and seawater, which would in turn be fed to filter feeding, bivalve molluscs. Theoretically such a practice would both effect tertiary sewage effluent treatment, through the removal of inorganic nitrogen and phosphorus nutrients, and produce a saleable end product, bivalve molluscs, whose market value would defray the cost of tertiary treatment. Subsequent studies (Goldman and Ryther, 1975) indicated that mass outdoor cultures of marine phytoplankton were indeed very efficient in effecting removal of waste nutrients; however, a major problem arose in that no simple means was found to control the predominant species of phytoplankton in such cultures. The diatom *Phaeodactylum tricornutum* was found to predominate over a wide temperature range in these highly eutrophic, light limited, continuous cultures, being displaced only at low temperatures by the diatom *Skeletonema costatum*, and occasionally at higher temperatures by green flagellates. *Phaeodactylum* has been compared to other phytoplankton species for its food value to both larval and juvenile bivalve molluscs. In general it has supported only poor or moderate growth (Davis and Guillard, 1958; Walne, 1970;

Epifanio *et al.*, 1975). Consequently it was not surprising to find that the two bivalve species originally cultured in the Woods Hole system, *Crassostrea virginica* and *Mercenaria mercenaria*, exhibited poor growth in this regime (Ryther, 1975).

Some progress was made during the 1975-1976 fiscal year when a comparison was made of the growth of six species of bivalve molluscs in the pilot scale waste recycling facility at Woods Hole. As in previous studies *C. virginica* and *M. mercenaria* grew poorly, however promising results were obtained with the Japanese oyster *Crassostrea gigas*, the European oyster *Ostrea edulis* and the Manila clam *Tapes japonica* (Mann and Ryther, 1977). More recently these findings have been confirmed and recorded growth rates improved through manipulation of physical environmental parameters (Mann and Glomb, 1978; Mann 1979). Both the mussel *Mytilus edulis* and the bay scallop *Argopecten irradians* have also been recently grown successfully in the Woods Hole facility (Ryther and Mann, 1977).

The ability to culture shellfish successfully using phytoplankton grown in sewage-seawater mixtures resulted in two new phases of the bivalve culture program at Woods Hole. These were (1) to attempt to optimize growth of the cultured species by control of the physical environment, and (2) to begin to examine public health implications of using sewage effluent in bivalve culture. The first phase, (1) above, was completed during the 1976-1977 fiscal year under a grant from the N.O.A.A. Office of Sea Grant (Ryther and Mann, 1977). Phase (2) was initiated in

May 1976 with the aid of a two-year grant from the Scaife Family Charitable Trusts. In the planning of this second phase various options were available for study. Secondary sewage effluent, depending upon source, has been shown to contain a variety of trace contaminants including metals, trace organics and human enteric viruses (see Chen *et al.*, 1974; Shuval, 1970). Due to both funding and practical limitations it was not considered feasible to attempt long term uptake and depuration of all three contaminant types simultaneously. Therefore, studies were initiated to examine uptake and depuration of a suite of trace metal contaminants (Cd, Cr, Cu, Hg, Ni, Pb, and Zn) by three species of bivalves (*C. gigas, O. edulis,* and *T. japonica*) over an 18 month period (an estimate of time required to reach market size from hatchery seed) using various time exposures to both sewage enriched and "clean", inorganically enriched phytoplankton cultures. By its very nature this study also provided an opportunity to culture seed stock to market size under defined environmental conditions for a prolonged period of time. A complete description of this study is given in Mann *et al.* (1978).

During the course of the trace metal contaminant study it became increasingly evident that problems would be encountered in any on-site application of a waste-recycling process due to the fluctuations in effluent quality. A major problem was the change in total suspended particulate load. It was not uncommon to obtain a tenfold

change in this parameter over a period of only a few days. It is highly desirable to minimize suspended particulate content of effluent in the present application for the following reasons:

1) To reduce variation in a major experimental quantity and thus allow greater confidence in resultant data and analysis.

2) As a considerable proportion of the trace metal, organic and virus content of polluted aquatic systems is associated with the suspended or sedimented particles; this represents a major source of contaminant in the proposed culture regime.

3) Due to their filter feeding habit bivalve molluscs will accumulate contaminants associated with particulate material with comparative ease.

Therefore efforts were made during the second phase of the contaminant-related studies to overcome this problem area. Under a grant from the NOAA, Office of Sea Grant for the fiscal year 1977-1978, a study was initiated to examine the use of simple, inexpensive low-technology methods for controlling effluent quality. A combination of a trickling sand filtration system with an activated charcoal column was used to remove particles, trace metals, and organic contaminants respectively prior to utilizing the effluent in algal culture. Programs were also initiated to assess the removal of contaminants by this system (metals and organic residues), and the rates and magnitude of uptake of these contaminants by shellfish exposed to phytoplankton cultured in sewage-seawater mixtures

using this "filtered" effluent.

Under the same grant, and through a co-operative program with the virology section of the Brookhaven National Laboratory, a program was initiated to examine long-term accumulation of viruses by *C. gigas* exposed to sewage enriched phytoplankton mixtures. This program involved the isolation and enumeration of viral contaminants from both sewage effluent and shellfish, and a series of depuration experiments on animals exposed to known doses of specific virus (vaccine strain Polio virus).

Literature Review

I. Trace metal contaminants in edible bivalve molluscs.

Trace metals in bivalve molluscs has been the subject of several recent excellent reviews (Bryan, 1971; Boyden, 1974, 1977; Fagerstrom, 1977; Phillips, 1977; Bryan and Uysal, 1978) to which the reader is referred.

Graham (1974) surveyed a number of species of bivalves from the southern California coastline for trace metal content and concluded that levels were strongly influenced by sewage discharge. However, O'Leary (1976) suggested that such influences may be minimal in areas where hydrographic conditions ensure rapid dilution and dispersal of effluent. Frazier (1976) recorded the seasonal changes in the trace metal content of *Crassostrea virginica* in the Rhode River of the Chesapeake Bay. He concluded that Mn, Fe and Cd contents of the soft tissues were significantly influenced by shell growth whereas Cu and Zn levels were influenced primarily by gonadal development and spawning.

Recently Bryan and Uysal (1978) have described the concentrations of ten metals in whole animals and specific tissues of the burrowing bivalve *Scrobicularia plana* in the Tamar estuary in England, in relationship to environmental levels of those same metals. Several major points are made which are of general interest to all bivalve mollusc-trace contaminant studies. These were:

(1) Metals partition between the organs of the mollusc, the

digestive gland often being the area of highest concentration (e.g. for Cd, Co, Cr, Ni, Pb and Zn).

(ii) Metals can be accumulated either directly from solution (e.g. Cu, Fe, Zn) or by ingestion with sediment or food.

(iii) Absolute body burden and concentration of metal may react differently to increasing body size e.g. concentrations of Cd, Co, Cr, Ni, Pb and Zn increase with body size, Fe remains relatively constant and levels of Ag, Cu and Mn decrease as body size increases.

Boyden (1977) reviews data on the effect of size on metal content of shellfish. Again, size dependent relationships were evident whereby concentrations decreased with increasing size (Zn in *Mytilus edulis*), were independent of size (Cd in *Mytilus edulis*), or increased with size (Cd in *Patella vulgata*). In general, relationships of the first kind, that is decreasing concentration with increasing size, exhibited an allometric relationship with weight, with an exponent value of approximately 0.7. Such relationships were constant within a single species of organism from comparable "clean" environments, but were sometimes subject to change in polluted areas (e.g. *Patella vulgata* exhibits higher exponent values for Zn and Cd in polluted areas whereas *Ostrea edulis* and *Mytilus edulis* do not). Fagerstrom (1977) discusses the potential physiological significance of such relationships and how this may be related to body burden and turnover time of these contaminants in marine organisms.

The significance of trace metal contents of bivalve molluscs is

further complicated by the effect of interaction of metal species on accumulation. Jackin *et al.* (1977) reported that the presence of Zn substantially depressed Cd uptake by *Mytilus edulis* as did a decrease in temperature. Similarly environmental fluctuations and behavioural responses can compound problems of interpretation. For example, *C. virginica* exposed to increasing concentrations of copper exhibited increased body burden at lower concentrations, but a constant body burden at higher environmental copper levels due to an associated decrease in pumping activity (F. L. Harrison, 1977 pers. comm.). Davenport (1977) has recorded periods of reduced pumping activity in *Mytilus edulis* in response to cyclical salinity fluctuations on a tidal periodicity suggesting that bivalve molluscs may be far from representative as environmental integrators of contaminant levels, despite their sessile, non-selective filter feeding habit in estuarine conditions.

Despite a rapidly increasing body of literature on the levels of trace metal contaminants in bivalve molluscs, the relationships between the level of contaminant that is measured in the mollusc, and that present in the environment from which it was collected is still poorly understood. In the context of waste recycling aquaculture systems certain problem areas relevant to this relationship are negated. For example sediments, which often form a sink for many trace metal contaminants in the coastal environment (see Phillips, 1977), are absent, hydrographic variability and tidal fluctuations are

eliminated, a continuous, quantifiable "contaminant" source is used, temperature is controlled to optimize growth, and controlled depuration periods can be easily affected. However, many poorly understood areas remain.

Very few controlled, long term, depuration studies have been performed to assess the time course of depuration in different species of bivalve in relation to physical parameters. Pringle *et al.* (1968) examined uptake and release of Pb by *C. virginica, Mercenaria mercenaria* and *Mya arenaria.* When transferred from contaminated to clean, flowing sea water depletion of the Pb content in *C. virginica* was faster than that observed for *M. mercenaria,* but slower than that observed for *Mya arenaria.* Also, there appeared to be direct relationships between uptake and depletion rates in all three species, suggesting that both activities were related to the same physiological processes, and the depletion rate and tissue level immediately prior to transfer to clean water. George *et al.* (1976) described the kinetics of accumulation and excretion of ^{59}Fe labelled ferric hydroxide in *Mytilus edulis* and its distribution in the tissues. A linear relationship, with no evidence of saturation effects, was recorded between the sea water concentration of Fe and all the tissues of the mussel. Partitioning between the tissues was evident with decreasing concentrations in the order viscera > kidneys > gills > muscle = mantle. On transfer to clean sea water some 35% of the Fe was lost through excretion and defecation, mainly the latter, during a period of seven days. However, approximately half of the re-

maining Fe was removed from the soft tissues of the mussel by transfer to the byssal threads.

The question of how bivalve molluscs effectively detoxify high body burdens of trace metal contaminants has long intrigued marine biologists. A recent study by George *et al.* (1978) has described the presence of granular amoebocytes containing extremely high (13,000 ppm Cu and 25,000 ppm Zn) concentrations of metals in *Ostrea edulis* from polluted waters. These granular amoebocytes are further compartmentalized from the oyster tissues in membrane limited vesicles which effectively reduced the trace metal levels "observed" by the oyster tissues.

The consumption of shellfish containing high levels of trace metal contaminants has been a cause for concern for many years. Perhaps the worst and most widely publicized example of human suffering that can be ascribed to consumption of contaminated fish and shellfish is Minamata "disease", severe neurological and developmental disorders related to mercury poisoning. At present the U.S. Food and Drug Administration enforce a maximum permissible level of 0.5 ppm wet weight of Hg in seafoods. This legislation is the source of considerable discussion in that Eisler (1977) considers this level too lenient for pregnant women whereas Carter (1977) considers the level over-cautious. Permissible levels of other metals have been examined since 1972 by the Shellfish Sanitation Branch of the Food and Drug Administration, and have been discussed at a series of open

workshops held annually since that date. Surprisingly legislation is still lacking in this area. However a set of "alert" levels have been proposed for five metals as being representative of "clean" shellfish grown in non-polluted areas. Levels exceeding "alert" levels have been suggested to be indicative of possible pollution. The present alert levels are summarized in Table 1.

Table 1. F.D.A. proposed "alert" metal concentrations in *Crassostrea virginica* and *Mercenaria mercenaria*.

Metal	Alert level ppm wet weight.	
	C. virginica	*M. mercenaria*
Cd	3.5	0.5
Cr	2.0	1.0
Cu	170	10
Pb	2.0	4.0
Zn	2000	65

II. Organic Contaminants in Bivalve Molluscs.

This subject has received much attention in recent years, stimulated by concern about oil spills, and domestic and industrial effluent discharge. Organic trace contaminants include literally thousands of distinct compounds and generalizations concerning their relationship with bivalve molluscs are not possible. The present review will focus on two groups of organic compounds common to effluent sources, the hydrocarbons and chlorinated biphenyls.

Anderson *et al.* (1974), Stegeman (1974) and Boehm and Quinn (1977) have reviewed the available literature on accumulation and depuration of hydrocarbons in a number of bivalve mollusc species. Certain consistent trends were evident. Stegeman and Teal (1973) exposed *Crassostrea virginica* to No. 2 fuel oil for a period of 49 days. On subsequent exposure to clean sea water over 90% of the accumulated hydrocarbon was eliminated in a period of 28 days. Anderson (1973) showed that *C. virginica* and *Rangia cuneata* were able to accumulate a wide variety of hydrocarbons in their tissues, but effected depuration in 10-52 days when transferred to clean sea water. A trend was evident in their experiments with napthalenes being consistently concentrated to the highest extent and requiring the longest period for depuration. Similar results were obtained by Cox *et al.* (1975) who exposed *C. virginica* to No. 2 fuel oil for 38 days. Fossato and Canzonier (1976) report the rapid elimination of hydrocarbons by *Mytilus edulis* previously exposed to clay-adsorbed diesel oil for a period of 41 days

although, as with the study of Stegeman and Teal (1973), a residual level of hydrocarbon in excess of that recorded for control animals was evident at the end of the depuration period.

Stegeman and Teal (1973) noted the strong association between accumulated hydrocarbons and the lipid content of the exposed molluscs. Both direct absorption and food associated ingestion of hydrocarbons are suggested as uptake mechanisms although their relative contributions have yet to be quantified (Stegeman, 1974).

Recently Boehm and Quinn (1977) attempted depuration of *Mercenaria mercenaria* which had been continually exposed to hydrocarbon pollution over a period of years. Only slight depuration (a reduction in concentration from 41.9 to 29.3 ppm wet weight total hydrocarbons) was evident over a period of 120 days. The authors suggest that the period of exposure to hydrocarbon may be instrumental in determining persistence of these compounds in bivalves in that over extended periods hydrocarbons may become increasingly associated with non-exchangeable sites in the organism.

Chlorinated biphenyls have been introduced into the marine environment over several decades in industrial effluent (e.g. from the electronics industry). These compounds exhibit low solubility and very long half lives in sea water. The latter property is of concern in that although such toxic PCB's as Aroclor 1254 are limited by legislation in their present usage, they have a continuing presence in the coastal environment (see Vernberg *et al.*, 1977 for recent literature).

Vreeland (1974) described the accumulation of a number of PCB's by *Crassostrea virginica* and concluded that concentration in the animal was directly related to environmental levels, and to the degree of chlorination of the isomer. PCB levels in the experimental animals stabilized in approximately one month. Vreeland (1974) noted that total body burden increased as the oyster grew, but absolute concentration of PCB remained constant.

Recently Langston (1978) reported that the clams *Cerastoderma edule* and *Macoma balthica* which had been previously exposed to 0.25 ppm Aroclor 1242, 1254 and 1260 for a period of ten days, were able to reduce their body burdens of these pollutants on transfer to clean sea water. Elimination rates decreased with increasing chlorination and removal of isomers with more than 5 chlorine atoms was not recorded. Isomers with "ortho"-substituted chlorine atoms were least persistent. Depletion was quicker at 15°C than at 8°C.

Courtney and Denton (1976) demonstrated that no depuration of PCB was evident over a six month period by *Mercenaria mercenaria*. PCB's, like hydrocarbons, appear to accumulate in tissue rich in lipid (Couch, 1975; Cahn *et al.*, 1977). Should these compounds exhibit similar properties to that suggested for hydrocarbons by Boehm and Quinn (1977) in being incorporated into non-exchangeable sites following prolonged exposure, then the general outlook for depuration of such compounds is bleak.

Present F.D.A. tolerance levels for PCB's in fish and shellfish products is 5 ppm although this is being reviewed and may possibly be reduced to 2 ppm.

III. Viruses in Shellfish

There has been increasing concern over the carriage of human viruses by shellfish. While there is little epidemiological evidence for the transmission of virus disease from the consumption of sewage-contaminated shellfish, (with the notable exception of infectious hepatitis), the potential for infection cannot be ignored. Fugate, Cliver and Hatch (1975) outlined a number of reasons why a potential health hazard exists: 1) shellfish raising waters are continually being subjected to high levels of pollution from sewage sources; 2) shellfish, being filter feeders, are able to efficiently concentrate viruses from the surrounding waters; 3) shellfish are frequently consumed raw or with minimal cooking which may not be sufficient to inactivate all of the viruses within them.

The occurrence of human virus (i.e., enterovirus) in various shellfish species is well documented. Morris, Mearns and Kim (1976), while studying the presence of virus in the California mussel found that 18 of the 39 samples tested contained virus. The mussels had been taken from beds located near outfalls which were discharging primary and secondary treated sewage effluent. Viral enumerations revealed concentrations ranging from 25 to 1475 PFU/kg of meat. Fugate *et al.* (1975) found virus in 2 of 17 oyster samples in Texas and 1 of 24 samples taken from the Louisiana Gulf Coast. The oysters had been taken from areas which had met the approved coliform standard. Virus species isolated included ECHOvirus type 4 and Poliovirus

type 1 from the Texas oysters, and Poliovirus type 3 from the Lousiana oysters. In 1968, Metcalf and Stiles isolated Poliovirus, Coxsackie B-3 and Reovirus from shellfish growing in a sewage-polluted estuary in New Hampshire. Coxsackie type A was isolated from 7 of 70 osyter samples and 2 out of 10 mussel samples found in a French market (Denis, 1973).

Although many enteric virus isolates have been found in shellfish, there is no epidemiological evidence to indicate that consumption of contaminated shellfish would lead to infection. There is, however, well-documented evidence for the shellfish-mediated transmission of infectious hepatitis. The first reported shellfish-related outbreak occurred in Sweden in 1955, resulting in 629 cases of infectious hepatitis (Roos, 1956). Since then, outbreaks have occurred in New Jersey, Mississippi and Alabama in 1961; in Philadelphia and Connecticut in 1963; in North Carolina in 1964; New Jersey in 1966; and in Rhode Island and Massachusetts in 1971 (Portnoy *et al.*, 1975). An outbreak occurred during October and November 1973 (Portnoy *et al.*, 1975) affecting 263 individuals from Houston and 15 from Calhoun, Georgia following the consumption of raw oysters from Louisiana Bay. After eliminating the possibility of contamination during transportation and storage, investigators concluded that the oysters were contaminated prior to, or at the time of harvesting. The area from which the oysters were harvested had been closed six weeks earlier due to contamination by polluted flood waters from the Mississippi

Valley. On September 1, the area was recertified using a coliform standard. The authors concluded that the Hepatitis virus had been retained with the oysters for periods of up to six weeks. More recently, Mahoney *et al.* (1976) detected the presence of Australia antigen (Au), indicative of the presence of type B Hepatitis virus, in Maine clams. The clams were taken from waters known to be contaminated with untreated sewage from a local hospital. It was found that the antigen could be transmitted to previously uninfected clams and indicated that shellfish could act as both vector and reservoir for Au antigen and type B Hepatitis virus.

DiGirolamo *et al.* (1977) proposed a mechanism for the attachment of virus to shellfish mucus during feeding. Utilizing a number of enteric viruses in seeded laboratory experiments, they found that virus particles became ionically bound to secretions. The binding sites were found to be the sulfate radicals in the mucopolysaccharides of the shellfish mucus. The uptake of virus particles by shellfish occurred rapidly resulting in the initial accumulation of large numbers of virus in the digestive gland of the animal. Unfortunately, this study did not attempt to detect any subsequent distribution of virus. Liu, Seraichekas and Murphy (1966a) found that 70% of the poliovirus seeded into seawater tanks were accumulated by the Northern quahog in 48 hours. DiGirolamo, Liston and Matches (1975) reported a similar rate of uptake in the West Coast oyster with 80 to 90% of the seeded viruses being accumulated within 24 hours. Liu,

Seraichekas and Murphy (1966b) found that maximum efficiency of virus uptake occurred when virus concentrations in the surrounding water were at low levels. Hamblet *et al.* (1969) reported that oysters subjected to low turbidity water accumulated three times as many poliovirus as oysters in high turbidity seawater.

Although high virus titers can be accumulated by shellfish, depuration can also be affected in clean, flowing seawater. Depuration rates have been found to be dependent on water temperature and salinity (Liu, Seraichekas and Murphy, 1967). These studies showed removal of virus occurring in 48 hours at 18°C. Reducing the temperature to 13°C resulted in an increase in the depuration time. Little or no depuration occurred at 8°C, a temperature at which the shellfish ceased pumping. The authors also demonstrated that a reduction of 50% in the salinity of the water was sufficient to halt the virus depuration process. Studies conducted in an estuarine environment by Vaughn and Metcalf (1975) showed that complete virus removal from seeded oysters required a period of 21-30 days in summer (17-22°C), and 60-80 days during winter months (-1-12°C). These results tended to confirm those of several earlier studies. Hamblet *et al.* (1969) concluded that under controlled environmental conditions, oysters can effectively eliminate virus irrespective of turbidity levels. The optimal conditions for depuration were judged to be: continuously flowing virus-free seawater of either high or low turbidity; a temperature optimum of 20°C; and a salinity of greater than 18 ppt.

In addition to determination of uptake and depuration rates, the question of virus survival within the shellfish has also been addressed. Morris *et al.* (1976) calculated that enteric viruses could survive in mussel tissue three to six times longer than coliform bacteria. Hedstrom and Lycke (1964) found Poliovirus to be more stable in oyster tissue than in the surrounding waters.

Physical-Chemical Options for the Reduction of Trace Contaminant and Virus Levels in Waste Water Effluents.

Conventional treatment of sewage effluent usually consists of the separation or settlement of primary solids, followed by secondary, biological oxidation prior to discharge. The general goal of such a procedure is to produce a clear liquor which is predominantly a solution of inorganic phosphorus and nitrogen. However, passage of viruses (Clark, 1956, 1961), metals associated with particulate material (Chen, 1973; Rohatgi and Chen, 1975; Davis and Jacknow, 1975) and organic residues and detergents (Hager and Flentje, 1965) through such systems is well documented. The use of effluent thus contaminated for enriching a food chain will obviously result in potential contamination of cultured organisms. Rather than have to resort to extensive depuration periods it would appear prudent to effect some tertiary treatment of effluent by physical-chemical means prior to use in food chain enrichment.

During the period 1968-1971 an experimental advanced wastewater treatment plant was operated at the South Tahoe Public Utility District in South Lake Tahoe, California to effect complete water pollution control through physical-chemical means on a large scale (7.5 mgd) (Anon, 1971). A summary of plant operations is described below to illustrate the effective, but expensive, present alternative to biological systems of treatment.

Following conventional activated sludge treatment lime is added

to effect coagulation of remaining solids and phosphate removal at the resultant high pH (11+). The flocculated material is subsequently separated and gaseous ammonia removed by spraying the resultant liquor down a vertical tower against a counter-current of air. Following a subsequent pH reduction an addition of alum is effected to further precipitate dissolved trace metals (see also Tatsumoto, 1978). These are removed by mixed media filtration. Final organic scrubbing is effected by granular activated charcoal adsorption. A final chlorination step is effected before discharge. Various of the components of the system can be reclaimed and recycled; granular carbon by thermal regeneration while lime mud is recalcined. All sludges are combusted to an inert, sterile ash.

Of particular interest in the context of waste recycling aquaculture systems is methodology for decreasing trace metal and organic levels, namely filtration and activated charcoal adsorption. A simple sand filtration - charcoal column was chosen for investigation in the present study as this offered the following advantages:

(i) effective removal of trace metals and organic compounds associated with particles or naturally occurring as particulate entities.

(ii) ease of operation, this being restricted to occasional backflushing of the sand filter.

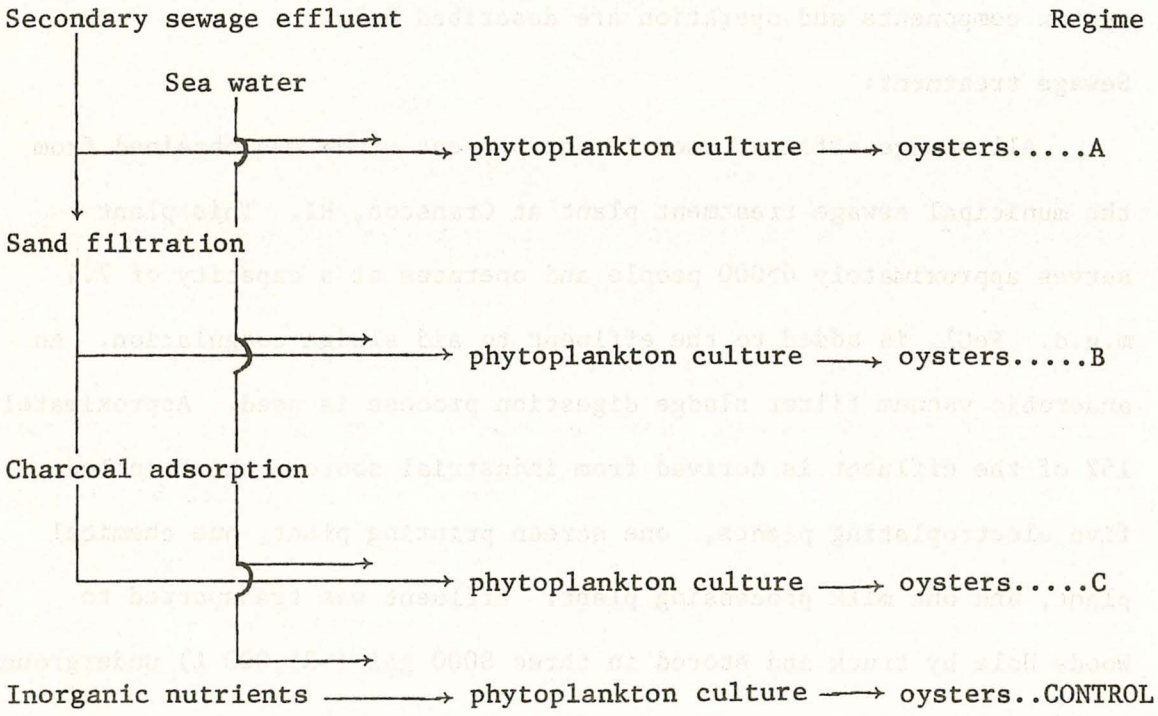
(iii) low cost.

Secondary benefits to this arrangement include the general enhancement of carbon adsorption by prior particulate removal (Anon., 1971), and

a decreased potential for virus survival due to a decreased dissolved organic carbon level in the effluent used for phytoplankton culture (see Vaughn and Ryther, 1974).

MATERIALS AND METHODS

The present study was designed to evaluate the effectiveness of various levels of simple, inexpensive, physical tertiary sewage treatment (sand filtration with and without activated charcoal adsorption) on the removal of selected trace metals and organics from the sewage effluent of an industrial urban area. Effluent was subsequently used to fertilize a marine aquaculture food chain. Operation may be summarized diagramatically thus:



Three levels of tertiary treatment were effected [(A) none, (B) sand filtration (C) sand filtration plus charcoal adsorption] with the effluent from each treatment fertilizing a separate food chain. A fourth

food chain (CONTROL) enriched with inorganic fertilizer served as a "zero pollution" control. The four regimes are hereafter referred to as A, B, C, and CONTROL.

The system was operated for 24 consecutive weeks. During weeks 0-12 the four regimes were operated as described above and oysters were periodically removed for assay for accumulation of selected contaminants. During weeks 13-24 all food chains were operated as per CONTROL to attempt depuration of accumulated contaminants. Again, oysters were periodically removed to assay depuration. Details of system components and operation are described below.

Sewage treatment:

All sewage effluent used in the present study was obtained from the municipal sewage treatment plant at Cranston, RI. This plant serves approximately 65000 people and operates at a capacity of 7.4 m.g.d. $FeCl_2$ is added to the effluent to aid sludge coagulation. An anaerobic vacuum filter sludge digestion process is used. Approximately 15% of the effluent is derived from industrial sources which include five electroplating plants, one screen printing plant, one chemical plant, and one milk processing plant. Effluent was transported to Woods Hole by truck and stored in three 8000 gal (∿35,000 ℓ) underground tanks until used.

Sand filtration was accomplished using a filter constructed from a linear polyethylene tank (1 m depth x 0.5 m diam). The filter outlet, at the base of the tank, was 5 cm below a perforated grid (10 mm diam

perforations) which supported the mixed-bed filter material. The latter consisted of 5 cm of gravel (mean size - 3ϕ), overlain by 5 cm of coarse sand (mean size 0ϕ), and a 40 cm surface layer of washed fine sand (mean size 2ϕ). An overflow port was situated 25 mm above the surface of the fine sand layer. Sewage flowed into the filter through a port 5 mm above, and diametrically opposite to the overflow port, at a rate of 250 ml/min. This was greater than the 210 ml/min outflow required for algal culture and ensured a constant head of liquid over the filter bed. The filter operated for 23½ hours per day, the remaining 30 minutes were employed in backflushing the filter with a 1/3 hp electric pump, operating through a restriction valve, to prevent build up filtered material at the filter bed surface. Except for regular backflushing no operational problems were evident in the filter system throughout a preliminary four week test period and the subsequent 12 week experimental period.

Effluent from the sand filter was divided equally into two flows of 105 ml/min. The first of these flowed directly to the phytoplankton culture of regime B. The second passed through a charcoal column, (25 cm length, 8.5 cm internal diameter) constructed from p.v.c. and plexiglass, and painted black to exclude light, before flowing into the phytoplankton culture of regime C.

Phytoplankton Culture

Each regime included one phytoplankton culture contained in a rectangular plywood tank 2.5 m L x 1.85 m W, fitted with a sloping

bottom (D increasing from 0.3 to 0.9 m) giving a nominal capacity of 4500 L. Phytoplankton cultures were aerated continuously by means of an air line fixed along the deepest edge of the tank. All cultures were operated on a continuous basis. In the sewage enriched regimes influent rates were 315 ml/min of seawater and 105 ml/min sewage. In the CONTROL regime seawater was added at the rate of 400 ml/min. with an accompanying enrichment of NH_4Cl and NaH_2PO_4 in the ratio 5 N:1 P being added at a rate of 20 ml/min to provide enrichment equivalent to that of the sewage (25% sewage $\triangleq$ 250 µg at /L N after dilution). "Harvest" phytoplankton overflowed through a standpipe and, by gravity, to the shellfish tanks.

Flow rates, cell concentrations and culture temperature were all monitored on a daily basis. All supply and harvest lines were cleaned twice per week or more frequently as required.

Throughout the experimental period no washout of cultures was observed at the dilution rate of 40%/day although cultures occasionally failed. In these latter situations cultures were restarted within 24 hours from reserve cultures maintained in 120,000 outdoor ponds. Cultures were maintained at ambient temperature throughout the study (3-18°C range). The diatom *Phaeodactylum tricornutum* was the dominant species in all regimes throughout this period.

Shellfish Culture

Crassostrea gigas were obtained as 5 mm seed from Sea Salter Shellfish Ltd., Whitstable, England in June 1976 and maintained in flowing

seawater, at ambient temperature, at Woods Hole Oceanographic Institution until commencing the present study in the fall of 1977. Forty-nine populations, each of 20 individuals (mean live weight 25-30 gms), were selected from the parent stock. Each population was transferred to a labelled plastic mesh tray (60 cm x 60 cm x 6 cm, Nestier Corp., Cincinnati; Ohio). Four groups of 12 populations were selected at random. Each group was transferred to a separate fiberglass-lined, wooden tank (3 x 0.75 x 0.75 m). The remaining population was sacrificed for assay of initial trace contaminant content as described later in the present text. Each of the tanks containing the experimental oyster populations was supplied with unfiltered seawater (30^{o}/oo salinity or greater), heated to 15^{o}C, at the rate of 8 L/min. Sea water flowed through the trays containing the oysters and to waste via a standpipe. Each tank also received the harvest of one of the previously described phytoplankton cultures (420 ml/min). Thus the four tanks containing the experimental oyster populations were equivalent to regimes A, B, C and CONTROL. Mixing of the tank contents was effected by gentle aeration from a perforated air-line fixed along the bottom of the tank. All tanks and associated pipes were cleaned at weekly intervals.

Sampling Program

(i) Sewage effluent

Sewage effluent (secondary treated, sand filtered and sand-plus-charcoal filtered) was sampled three times per week for both trace

metal and organics. Samples for trace metal analysis were collected in an acid-washed, glass beaker and filtered through an acid-leached, 25 mm Gelman A/E glass fibre filter. The filters were stored under dessication until subsequent analysis of the retained particulate material. The filtrate was stored in L.P.E. bottles at $4^{o}C$ following acidification (1 ml conc HNO_3 per 100 ml filtrate) to await assay.

Four litre quantities of the effluents used in culture regimes A and C were collected three times per week and stored at $4^{o}C$, in hexane washed bottles, to await analysis for organics. Assay was effected at the Harold E. Edgerton Research Laboratory of the New England Aquarium under the supervision of Dr. T. Gilbert.

(ii) Shellfish

At intervals of two weeks throughout the 24 week duration of the experiment four experimental populations of oysters, one from each of the culture regimes, was removed for contaminant assay. Animals were counted and any mortalities recorded prior to weighing to estimate mean live weight. Of the 20 individuals in each population, five were shucked and freeze-dried to await trace metal assay for Cd, Cr, Cu, Pb, Ni and Zn, five were deep frozen to await assay for Hg, and the remainder were frozen to await assay for organics. Regime B was not assayed for organics; these specimens were shucked, preserved in Bouins fixative, and subsequently examined histologically for evidence of gonad development (see Mann,1979,) to investigate the possibility that the high lipid contents associated with gametogenesis

may influence contaminant accumulation.

Contaminant Assay

(i) Trace metals

Cu, Zn and Cd contents were sufficiently high in the dissolved phase in effluents to be determined directly by flame atomic absorption. Dissolved Ni, Pb, and Cr levels were analyzed using a Perkin-Elmer 303 atomic absorption spectrophotometer equipped with a graphite furnace. Filters containing sewage particulate material were ashed at 550°C for 30 minutes, boiled with "Ultrex" nitric acid and the leachate volumetrically fixed. Cd, Cr, Cu, Ni, Pb and Zn were subsequently analyzed by the graphite furnace technique described previously.

Freeze dried oyster tissue was ashed at 550°C, digested with "Ultrex" nitric acid, filtered and volumetrically fixed for analysis. A duel channel atomic absorption spectrophotometer was used to determine the high salt background effect during analyses by flame. Determinations were attempted using a graphite furnace, but specific salt interferences necessitated the use of salt matched standards, method of additions and a great deal of time which made its use impractical for the freeze-dried samples. Flame analysis showed no background effect for Cu or Zn. Cd, Pb and Ni displayed a signal enhancement with salt. This was corrected by monitoring the background on the secondary channel using a nearby non-absorbing line and electronically subtracting the background from the primary signal. The Cr signal was constantly inhibited above 1000 ppm salt. Since the inhibition cannot

be electronically corrected, standards were used containing 1200 ppm of the nitrate species of the major salts.

As Hg is subject to volatolization at low pressure freeze drying was inadequate for sample preservation in the present study. Assays were made on fresh or fresh-frozen tissue and results corrected for water content using data collected during freeze drying.

Mercury values were obtained using closed system, cold vapor generated atomic absorption preceded by acid - permanganate digestion of the sample. Absolute concentration was measured with digested standards while a two point method of additions was employed to determine any matrix effect.

PROCEDURE FOR THE MEASUREMENT OF HYDROCARBONS AND CHLORINATED HYDROCARBONS (ORGANICS) IN SEWAGE SAMPLES AND OYSTER TISSUES.

A. Sewage Sample Preparation

Liquid samples of raw and treated sewage were filtered through precleaned Metrigard superfine glass fiber filters. One liter of the filtrate was then extracted three times with 25 ml portions of hexane. The combined hexane extracts were dried over Na_2SO_4, concentrated to about 5 ml and chromatographed on a silica gel column.

The solids collected on the glass fiber filters were air dried overnight, weighed and extracted with hexane for 16 hours in Soxhlet apparatus. The extract was concentrated to about 5 ml and chromatographed on a silica gel column.

B. Oyster Tissue Preparation

About 10 oysters were shucked and the soft parts combined and homogenized in a blender. Ten grams of tissue were digested overnight with 4 N NaOH at 90°C, and then extracted three times with 15 ml ethyl ether. The combined ether extracts were dried over Na_2SO_4, and concentrated to ∿1 ml. Two ml of hexane were added to the extract and the extract reconcentrated to 1 ml. This hexane extract was chromatographed on a silica gel column.

C. Silica Gel Column Chromatography

All samples were chromatographed using a 0.9 x 25 cm column filled with 5 gms silica (100 mesh, activated to 150°C, and then deactivated with 3% H_2O). After the column had been washed with hexane, the sample was added to the top and eluted with 25 ml hexane. The fraction collected

(A) contains essentially all the Arochlor 1254 components and the non-aromatic hydrocarbon components. The column was then eluted with 25 ml 6% ethyl ether in hexane. This fraction (B) contained the aromatic hydrocarbon components. Both fractions of each extract were analyzed by gas chromatography.

D. Gas Chromatographic Measurements

Determination of chlorinated hydrocarbons was performed using a 2 m. glass column packed with 3% SE-30 on Chromosorb WHP and a ^{63}Ni electron capture detector. Separation conditions were isothermal operation at 180°C with injection port and manifold temperatures of 225°C and a detector temperature of 250°C. The carrier gas was 5% methane in argon. Samples were compared with Arochlor 1254 standards to determine concentration.

Petroleum hydrocarbons were determined using a 2 m. stainless steel column of 3% Dexsil 300 on Chromosorb WAW and flame ionization detection. Separation conditions were column temperature programming with an initial temperature of 100°C, held for four minutes. Injection port was at 250°C and the manifold at 300°C. The carrier gas was 99.999% helium. Dual columns were used with the FID. Samples were concentrated ∿100 times prior to injection and compared with standard concentrations of a normal alkane series C_{10} to C_{30}.

RESULTS

Trace Metals in Sewage Effluent.

Trace metal levels in both the dissolved (D) and the particulate (P) phases of the Cranston effluent for each of the three culture regimes are summarized by sampling intervals in Table 2. Detectable levels of mercury (Hg) were evident only in the dissolved fraction.

A consistent decrease in particulate metal concentrations following sand filtration is evident in all of the metals assayed. The resultant effluent was consistently below 0.1 ppm Cd (excepting one instance), 10 ppm Cr, 4.2 ppm Cu, 2.0 ppm Ni, 2.0 ppm Pb (excepting two instances) and 5.6 ppm Zn. Consistent significant decreases in dissolved metal levels were only evident for Cd and Cr following sand filtration. Summarizing data for both phases in Table 3 it is evident that marked decreases in overall metal contents were evident for Cd, Cr, Cu, Pb and Zn. Ni levels were consistently high in the present study as they were in previous studies of Cranston effluent. This is undoubtedly related to the presence of electroplating plants discharging their effluent in the Cranston treatment facility. However, contaminant levels in the effluent used in the present study are still well below maximum permissible levels for drinking water (Table 3) suggesting efficient operation of the Cranston sewage treatment plant.

Table 2: Dissolved (D) and Particulate (P) metal concentrations in Cranston sewage effluent during the experimental period. All values (ppb) are the mean of at least three samples within the time period. Regimes are A: secondary treated; B: secondary treated plus sand filtration; C: as for B plus activated charcoal filtration. * indicates contaminated sample.

		Metal and Regime																				
		Cd			Cr			Cu			Hg			Ni			Pb			Zn		
Weeks	Phase	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
0- 2	D	0.43	-	-	3.3	1.6	1.5	8	26	18	<.3	<.3	<.3	123	129	136	1.2	0.83	0.76	25	31	24
	P	0.28	<.05	<.05	6.6	<1	<1	60	2.0	<2	-	-	-	20	1.3	1.5	8.6	1.7	<1	30	3.7	2.0
2- 4	D	0.51	-	-	2.2	1.8	2.0	8	11	28	<.3	<.3	<.3	99	145	331	2.1	0.91	0.87	23	22	63
	P	1.4	<.05	<.05	30	<1	<1	332	<2	<2	-	-	-	83	<1	1.7	23	6.1	1.2	120	2.6	<2
4- 6	D	0.68	0.18	0.16	1.7	1.6	1.6	11	10	11	<.3	<.3	<.3	156	147	182	0.24	0.40	0.15	33	38	*
	P	0.65	0.13	0.08	5.7	<1	<1	53	<2	2	-	-	-	15	2.0	<1	15	4.6	1.9	24	5.6	<2
6- 8	D	0.43	0.34	0.22	1.4	1.2	1.3	6	8	20	<.3	<.3	<.3	149	151	191	0.09	0.19	0.06	32	38	36
	P	0.84	0.06	<.05	4.3	<1	<1	74	<2	<2	-	-	-	38	<1	<1	8.1	<1	<1	41	<2	<2
8-10	D	0.47	0.12	0.33	1.4	1.5	1.5	11	11	25	<.3	<.3	<.3	156	162	166	0.40	0.09	0.12	4	27	38
	P	1.2	<.05	0.15	8.1	<1	<1	46	<2	4.2	-	-	-	19	<1	1.8	3.9	<1	1.7	39	<2	2.8
10-12	D	0.47	0.12	0.33	1.4	1.5	1.5	11	11	25	<.3	<.3	<.3	156	162	166	0.40	0.09	0.12	4	27	38
	P	1.2	<.05	0.15	8.1	<1	<1	46	<2	4.2	-	-	-	19	<1	1.8	3.9	<1	1.7	39	<2	2.8

Table 3. A comparison of trace metal levels in sewage effluents and seawater from the present study with the data of Jacobs (1973) for Cranston sewage; Mann *et al.* (1978) for Wareham and Cranston sewage, and E.P.A. standards for drinking water and marine waters discharge.

	Metal Content ppb Total						
	Cd	Cr	Cu	Hg	Ni	Pb	Zn
Present study A	1.42	12.36	110.3	<0.3*	172.7	5.46	69.0
Present study B	0.32	3.56	14.8	<0.3*	179.3	2.99	33.5
Present study C	0.26	2.56	23.9	<0.3*	196.8	1.76	46.0
Jacobs (1973) Cranston	----	<20	5.3	----	358	<20	159
Mann *et al.* (1978) Cranston	0.18	7.2	14.3	<0.3	61.0	3.8	15.3
Mann *et al.* (1978) Wareham	0.39	3.3	58.0	<0.1	9.4	1.7	56.3
Sea water present study	**	0.3	2.0	**	3.3	1.5	3.3
E.P.A. standards 1976 drinking water	10	50	1000	2	+	50	5000
Seawater discharge	5	+	+	0.1	+	+	+

*dissolved fraction only.

**below detection.

--not measured.

+no standard set.

Prior to discussing results of metal assay on the experimental populations of oysters (Table 4) it is relevant to note the serial dilution of sewage effluent during the culture regime used in order that metal levels given in Table 2 can be related to those in Table 4. Phytoplankton culture regimes A, B and C were enriched in the ratio 25% sewage effluent - 75% sea water. The resultant culture flowed into the shellfish holding tanks at a rate of 420 ml/min and was diluted by an accompanying flow of sea water of 8L/min. These flow rates were chosen to effect sufficient dilution of the cultured *Phaeodactylum tricornutum* from a concentration of 1×10^6-5×10^6 cells/ml to a concentration more equitable with optimum feeding concentrations in *C. gigas* i.e. 5×10^4-2.5×10^5 cells/ml. However, this dilution also resulted in a concentration of sewage effluent in the shellfish holding tanks equivalent to 1.25% sewage-in-seawater. These values are lower than those described for previous waste-recycling studies (e.g. Ryther *et al.* 1975) where concentrations of effluent in excess of 50% were examined for phytoplankton culture. However, providing phytoplankton cultures can be maintained in a light limited regime there is little to be gained from the use of exceedingly high sewage effluent concentrations for two reasons. Firstly, excess nutrient will only be passed through the culture system thus placing greater emphasis on the need for efficient cleansing mechanisms prior to final discharge e.g. macrophyte culture. Secondly, contaminants associated

Table 4: Mean metal contents (ppm) of *Crassostrea gigas* during exposure to sewage enriched food chains (weeks 0-12) and subsequent depuration (weeks 12-24). Regimes; A: secondary effluent; B: as for A plus sand filtration; C: as for B plus activated charcoal filtration; D: CONTROL.

	Metal Regime																							
	Cd				Cr				Cu				Hg				Ni				Pb				Zn			
Week	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
0				3.7				1.2				145				0.20				1.2				<.02				1050
2	3.4	3.7	3.5	3.7	1.2	1.2	1.6	1.2	397	131	199	151	0.31	0.24	0.30	0.20	1.2	3.6	3.8	1.9	1.6	0.11	<.02	<.02	1351	858	1152	1070
4	4.0	3.8	3.7	4.3	1.6	1.6	1.6	1.2	272	103	369	379	0.32	0.49	0.31	0.24	3.2	0.7	1.6	1.8	0.85	0.78	<.02	<.02	1160	798	1577	1509
6	4.6	4.1	3.5	3.6	1.7	1.3	1.6	1.3	299	221	74	108	0.26	0.33	0.18	0.21	5.3	1.3	1.7	1.4	<.02	0.32	0.29	<.02	1463	1074	938	1063
8	4.4	3.9	3.7	4.5	1.1	1.8	1.4	0.9	259	84	184	185	0.23	0.18	0.23	0.28	8.4	2.2	0.7	5.4	<.02	1.9	<.02	<.02	1250	874	1290	1296
10	3.5	2.0	4.5	4.7	1.5	1.1	1.2	1.4	159	86	148	212	0.22	0.18	0.24	0.15	1.1	0.8	1.4	0.9	0.67	0.74	0.34	0.48	1005	731	1053	1196
12	3.2	2.3	2.6	4.5	1.2	1.0	1.3	1.6	286	60	156	154	0.31	0.15	0.14	0.17	1.0	0.9	0.6	4.6	0.89	0.60	0.39	0.53	1138	543	855	1138
14	3.6	4.2	4.1	4.6	1.6	1.6	1.4	2.2	235	110	154	161	0.19	0.25	0.21	0.37	1.3	1.2	1.0	1.6	0.98	0.53	<.02	0.85	1022	809	878	1089
16	3.7	3.7	3.2	4.9	0.9	2.1	1.0	1.3	300	138	154	182	0.36	0.28	0.60	0.23	7.0	3.4	0.7	3.0	<.02	0.60	0.61	0.20	1119	879	825	881
18	4.0	4.4	4.5	5.6	1.8	4.3	0.9	1.4	222	133	171	140	0.33	0.25	0.12	0.17	1.3	1.4	0.7	1.0	0.28	0.35	0.73	0.29	892	736	810	745
20	3.8	3.7	3.1	4.2	2.2	1.1	0.9	1.7	251	108	98	148	0.22	0.18	0.17	0.26	0.8	0.9	0.6	0.7	0.11	0.23	0.23	0.05	928	652	570	839
22	3.3	3.1	3.3	3.3	1.2	1.0	0.8	0.8	317	74	120	110	0.28	0.17	0.14	0.14	0.6	0.6	0.3	0.4	0.60	0.79	0.36	0.23	1045	551	610	674
24	3.8	1.9	2.6	3.0	0.7	0.9	0.8	0.7	180	110	77	138	0.25	0.10	0.16	0.14	0.2	0.5	0.4	<.03	<.02	0.36	0.30	0.23	792	549	468	595

with the influent sewage will be presented to the cultured organisms in greater concentrations thus increasing the potential requirement for depuration.

Examination of the relative metal contents of the sea water and sewage used in the present study (Table 3), in conjunction with the relative volumes of each presented to the experimental oysters, reveals that in all instances it was the sea water, not the sewage effluent that contributed the major proportion of the total quantity of metal in all regimes. Consequently little variation would be expected in the metal contents of oysters from each regime. This is in fact the case in the present study.

Trace Metals in *Crassostrea gigas*

The F.D.A. "alert" levels for Cd in *C. virginica* is 3.5 ppm wet wt (*ca.* equal to 23.3 ppm dry wt). Levels for *C. gigas* in the present study vary between 1.9 and 5.6 ppm dry wt with no significant differences being evident between the experimental regimes, and either before or during "depuration". Cd exhibits cumulative retention in human liver and kidney tissue, and is causative of neurological disorders; however, the present data suggest no public health hazard from Cd contamination in the present instance.

The presence of Cr, like Cd, in marine waters is mainly derived from pollution associated with the electroplating industry. Hexavalent

Cr is toxic when inhaled, however the toxicity of ingested Cr (tri or hexavalent) is not known. Recorded Cr levels in *C. gigas* vary in the range 0.7-2.2 ppm dry wt and exhibit no marked differences temporally or between culture regimes. All values are below the F.D.A. alert levels for Cr in *C. virginica* (2.0 ppm wet wt, *ca.* equal to 13.3 ppm dry wt).

Levels of Cu in *C. gigas* were generally higher in regime A (159-397 ppm dry wt) than regimes B, C and CONTROL (77-221 ppm dry wt with the exception of CONTROL week 4). No temporal pattern was evident suggesting that "depuration" did not occur. The elevated levels in regime A are probably related to elevated Cu levels in the sewage effluent used in this regime (Tables 2 and 3); however, the magnitude of the difference between the oyster groups is reflective of the considerable dilution of the sewage as previously discussed. All recorded levels for oyster tissues are below the F.D.A. alert level of 175 ppm wet wt (*ca.* 1167 ppm dry wt).

Alkyl mercury compounds are very toxic to man (lethal dose 20-30 mg). Thus, acceptable Hg levels in foodstuffs are particularly stringent (0.5 ppm wet wt, *ca.* 3.3 ppm dry wt in shellfish). Levels recorded for *C. gigas* in the present study are in the range 0.10-0.49 ppm dry wt.

No limits have been set for permissible levels of Ni in either drinking water or shellfish as Ni is considered to be of low toxicity

to man. A single value of 8.4 ppm dry wt was recorded in *C. gigas* in regime A at week 8. Excepting this one instance all values were below 5.4 ppm dry wt, recorded in the control population at week 8.

Pb levels in *C. gigas* varied in the range <0.02-1.9 ppm dry weight but exhibited no obvious temporal pattern or differences between experimental regimes. The F.D.A. "alert" levels for oysters is particularly stringent at 2.0 ppm wet wt (13.3 ppm dry wt) as Pb is notably toxic causing gastro-intestinal problems, anorexia, abdominal pain, paralysis and anemia.

Zn is considerably less toxic than Cd, Cr, Hg or Pb. Adult humans require 10-15 mg/day. Recorded levels for *C. gigas* of 468-1577 ppm dry wt are well below the shellfish alert level of 2000 ppm wet wt (13,300 ppm dry wt).

Hydrocarbons in Sewage Effluent

Hydrocarbon concentrations in the liquid fraction were consistently below detection. In the particulate fraction (Table 5) it is evident that a consistent and considerable decrease in concentration in both the alkane (X) and aromatic (Y) fractions is evident following passage of the effluent through a sand filter and activated charcoal column. Temporal variability in the secondary effluent (regime A) is related to changes in suspended sediment loading; however, this variability is damped by the supplementary treatment applied to regime C. Overall levels are generally low, rarely exceeding 20 ppb

Table 5. Hydrocarbon levels in sewage effluent enrichment in regimes A and C during weeks 0-12. X: alkane fraction C_{20}-C_{30}, Y: aromatic fraction C_{20}-C_{30}, all values in ppb and for <u>particulate phase only</u>, liquid fraction consistently below detection limit (0.1 ppb).

		Regime A			Regime C		
Week	Date	X	Y	X+Y	X	Y	X+Y
0							
	9/22	0.5	*	0.5	*	*	*
	9/26	1.5	6.1	7.6	3.9	4.5	8.4
	9/28	3.9	*	3.9	1.4	*	1.4
	9/30	36.5	19.4	55.9	3.6	*	3.6
	10/3	20.3	70.5	90.8	5.0	0.8	5.8
2							
	10/5	16.8	11.4	28.2	0.2	0.1	0.3
	10/7	0.5	1.7	2.2	0.1	*	0.1
	10/10	10.8	4.5	15.3	*	0.1	0.1
	10/12	0.1	2.2	2.3	*	0.2	0.2
	10/14	2.3	8.1	10.4	4.8	0.5	5.3
	10/17	0.6	7.1	7.7	0.4	0.5	0.9
4							
	10/19	0.3	17.1	17.4	0.8	*	0.8
	10/21	0.8	12.1	12.9	----	----	----
	10/24	0.3	2.2	1.5	*	*	*
	10/26	0.6	1.3	1.9	*	0.6	0.6
	10/28	1.0	*	1.0	*	3.3	3.3
	10/31	*	1.3	1.3	0.3	1.2	1.5
6							
	11/2	1.7	4.8	6.5	0.4	1.6	2.0
	11/4	2.3	3.9	6.2	*	1.3	1.3
	11/7	2.6	4.2	6.8	3.2	6.9	10.1
	11/9	5.1	1.5	6.6	1.7	0.6	2.3
	11/14	7.3	7.5	14.8	*	0.2	0.2
8							
	11/16	2.2	3.5	5.7	24.8	2.5	27.3**
	11/23	9.0	8.4	17.4	*	0.3	0.3
10							
	11/30	3.9	8.2	12.1	----	----	----
12							

*below detection limit

**suspected contamination

in regime A and, with one exception, 10 ppb in regime C.

Hydrocarbons in *Crassostrea gigas*

Three major conclusions can be made from the data given in Table 6; they are (i) no trends are evident to suggest increasing hydrocarbon content in a sewage enriched regime or depuration of hydrocarbon content on transfer of oysters from a sewage-enriched to a clean regime. (ii) temporal fluctuations in all these regimes (A, C and CONTROL) appear to be random events. (iii) hydrocarbon levels are comparable in oysters grown in all three regimes suggesting that the measured quantities are, in fact, background levels inherent to the experimental stock and are not related to accumulation of hydrocarbon introduced with the sewage effluent enrichment.

P.C.B.'s in Sewage Effluent

The particulate phase consistently contributed the greater proportion of the total P.C.B. in the sewage effluent used in both regimes A and C throughout the study (Table 7). P.C.B. concentrations were consistently higher in the particulate phase in regime A, but concentrations in the aqueous phase were comparable for both regimes. The E.P.A. 1976 drinking water standards' maximum permissible level of 1ng/L is consistently exceeded by effluents used in both regimes A and C.

Table 6. Hydrocarbon levels in *C. gigas* in regimes A, C and CONTROL Week 0-12, sewage enrichment in regimes A and C Weeks 12-24, sewage free regime X: alkane fraction C_{20}-C_{30}. All values in ppb wet wt.

	A			C			CONTROL		
Week	X	Y	X+Y	X	Y	X+Y	X	Y	X+Y
0	4.4	7.8	13.2						
2	2.0	5.0	7.0	0.8	1.0	1.8	0.6	1.9	2.5
4	*	7.2	7.2	*	0.7	0.7	2.4	8.0	10.4
6	0.3	2.3	2.6	*	*	*	2.5	1.0	3.5
8	*	1.2	1.2	1.5	2.4	3.9	6.8	2.0	8.8
10	*	*	*	0.9	1.6	2.5	1.9	16.5	18.4
12	0.4	43.1	43.5	1.1	6.2	7.3	*	0.1	0.1
14	2.4	2.8	5.2	*	1.5	1.5	0.3	0.5	0.8
16	4.3	13.8	18.1	0.2	7.0	7.2	0.2	0.3	0.5
18	8.9	4.8	13.7	2.9	7.3	10.2	4.4	5.3	9.7
20	0.5	9.7	10.3	9.2	3.1	12.3	8.0	17.4	25.4
22	2.8	3.7	6.5	1.7	3.0	4.7	2.8	5.3	8.1
24	0.4	5.9	6.3	1.9	0.7	2.6	1.3	4.4	5.7

*below detection (0.1 ppb).

Table 7. P.C.B. levels in sewage effluent enrichment in regimes A and C during weeks 0-12. All values in ng/L efficient as Arochlor 1254.

Week	Date	Regime A		Regime C	
		Particulate	Aqueous	Particulate	Aqueous
0					
	9/22	13	<8	11	<10
	9/26	122	<1.2	22	<1.3
	9/28	71	<0.4	<4	<1.7
	9/30	1204	<1.0	<7	<1.2
	10/3	1155	<1.6	<7	<1.6
2					
	10/5	1575	<9	22	<2
	10/7	50	<4	--	<2
	10/10	94	<4	33	<8
	10/12	57	<8	40	<8
	10/14	118	<9	21	<8
	10/17	22	<2	10	<2
4					
	10/19	42	<3.4	14	<9
	10/21	60	<5	--	---
	10/24	154	<5	20	<10
	10/26	74	<5	14	<9
	10/28	61	<9	20	<9
	10/31	44	<9	25	<9
6					
	11/2	48	<8	14	<2
	11/4	71	<4	17	<2
	11/7	115	<2.1	<6	<1.7
	11/9	229	<3.1	<7	<1.7
	11/14	91	<1.2	--	<2
8					
	11/16	88	<6	20	<5
	11/23	187	<10	40	<2
10					
	11/30	97	<5	--	---
12					

Table 8. P.C.B. levels in *C. gigas* in regimes A, C, and D (control). Weeks 0–12; sewage enrichment in regimes A and C. Weeks 12–24; sewage-free. All values are given as ppb wet weight Arochlor 1254.

Week	A	C	D
0	51		
2	27	28	17
4	19	27	20
6	14	22	23
8	<2	27	49
10	<2	17	25
12	24	34	9
14	51	89	29
16	63	48	66
18	79	66	40
20	22	72	33
22	47	17	78
24	48	56	11

P.C.B.'s in *Crassostrea gigas* (Table 8)

The three conclusions stated with respect to hydrocarbons in *C. gigas* are equally applicable to the results of P.C.B. assay in the experimental *C. gigas*. There is no evidence of any accumulation and/or depuration throughout the time course of the experiment, random temporal variation occurs in the recorded levels, and no consistent differences are evident between the three regimes. Recorded P.C.B. concentrations are well below the proposed maximum tolerance of 2 ppm P.C.B. as reported in the Federal Register on April 1, 1977.

VIRUS STUDIES

INTRODUCTION

Of utmost concern is the presence of pathogenic human viruses in the sewage effluent (Trask *et al.*, 1938; Kollins, 1966; Berger 1976) and their potential threat to the social and legal acceptability of the products of the aquaculture system. Previous studies have shown that viruses are readily accumulated by filter-feeding mollusks (Metcalf and Stiles, 1965; Metcalf *et al.*, 1972; Liu *et al.*, 1966; Duff, 1967), and that ingestion of these contaminated shellfish by humans may result in disease (Roos, 1956; Taylor *et al.*, 1966; Mosley 1967). The ability of viruses to withstand the rigors of a sewage-seawater environment and rapidly growing algal cultures, and their resultant contamination of shellfish species would present a serious impediment to any commercial development of a sewage-aquaculture system.

In order to better understand the relationships between viruses and shellfish being propagated in the aquaculture system, a one-year study was initiated in 1977. Several related aspects were studied including 1) development and comparison of more effective methods for the extraction of viruses from oysters; 2) depuration of poliovirus and bacteriophage MS-2 by actively growing shellfish in the aquaculture system; 3) survival rates of MS-2 and a recently isolated non-vaccine strain of poliovirus in shellfish-growing waters. Several of these aspects will be further pursued under the auspices of a re-

search contract with the Food and Drug Administration.

METHODS AND MATERIALS

A) *Virus stocks*. Plaque-purified poliovirus type 1 (LSc) was obtained from the Baylor College of Medicine. Virus was routinely propagated on low-passaged Buffalo Green Monkey kidney cells (BGM-Microbiological Associates) and prepared by the procedure of Jakubowski *et al.* (1975). A non-vaccine (t⁻) strain of poliovirus type 3 was isolated in our laboratory from tertiary treated sewage effluent (Vaughn *et al.*, 1978). The virus was propagated and prepared as previously described. Cultures of bacteriophage MS-2, on RNA-containing coliphage (Strauss and Sinshaimer, 1963) and its host bacterium *Escherichia coli* p4x6 were obtained from Dr. R. M. Zsigray, Department of Microbiology, University of New Hampshire.

B) *Virus assay*. Samples for human virus assay were treated with chloroform for 30 min. and diluted in phosphate-buffered saline (pH 7.2). A 0.5 ml volume of sample was inoculated onto monolayers of BGM cells in T-25 flasks. Viruses were allowed to adsorb for 60-90 minutes at 30°C with gentle rocking. Inoculum was then decanted and 4 ml of a neutral red agar overlay (Hill *et al.*, 1976) added to each flask. Test flasks were incubated at 35°C and observed for plagues over a 10-day period. Bacteriophage-containing samples were enumerated using the agar-overlay technique of Adams (1959).

C) *Extraction and enumeration of virus from shellfish*. Human virus

extraction from shellfish involved a multi-step procedure (Figure 1) developed by Sobsey *et al.* (1975, 1978). In the latter paper, the authors reported that the time-consuming ultrafiltration step could be replaced by an acid precipitation with no loss of virus. All experiments reported to date by these authors have involved the seeding of oyster homogenates with known concentration of virus. No data has yet appeared with regard to viruses which have been accumulated naturally by shellfish from the surrounding waters. Studies were initiated in this laboratory to compare the virus extraction efficiency of the acid precipitation method versus the ultra-filtration method using oysters (*Crassostrea gigas*) which had accumulated seeded poliovirus type 1 from seawater. Bacteriophage enumerations from shellfish (*C. virginica, Mercenaria mercenaria*) involved the use of homogenates of pools of 10-12 animals (Vaughn and Metcalf 1975). Supernates recovered following clarification of homogenates were clarified by centrifugation for 10 minutes at 5000 xg and treated with assay as previously described.

D) *Shellfish depuration studies*. Three species of bivalves were used in the depuration experiments. *C. virginica* and *M. mercenaria* (Northern quahog) were used in bacteriophage studies, while *C. gigas* was used in the poliovirus depuration experiments. Young, actively growing oysters were collected from stocks already present in the Woods Hole laboratory, *M. mercenaria* were gathered from Waquoit Bay, Falmouth, Massachusetts. Shellfish were placed in circular basins containing

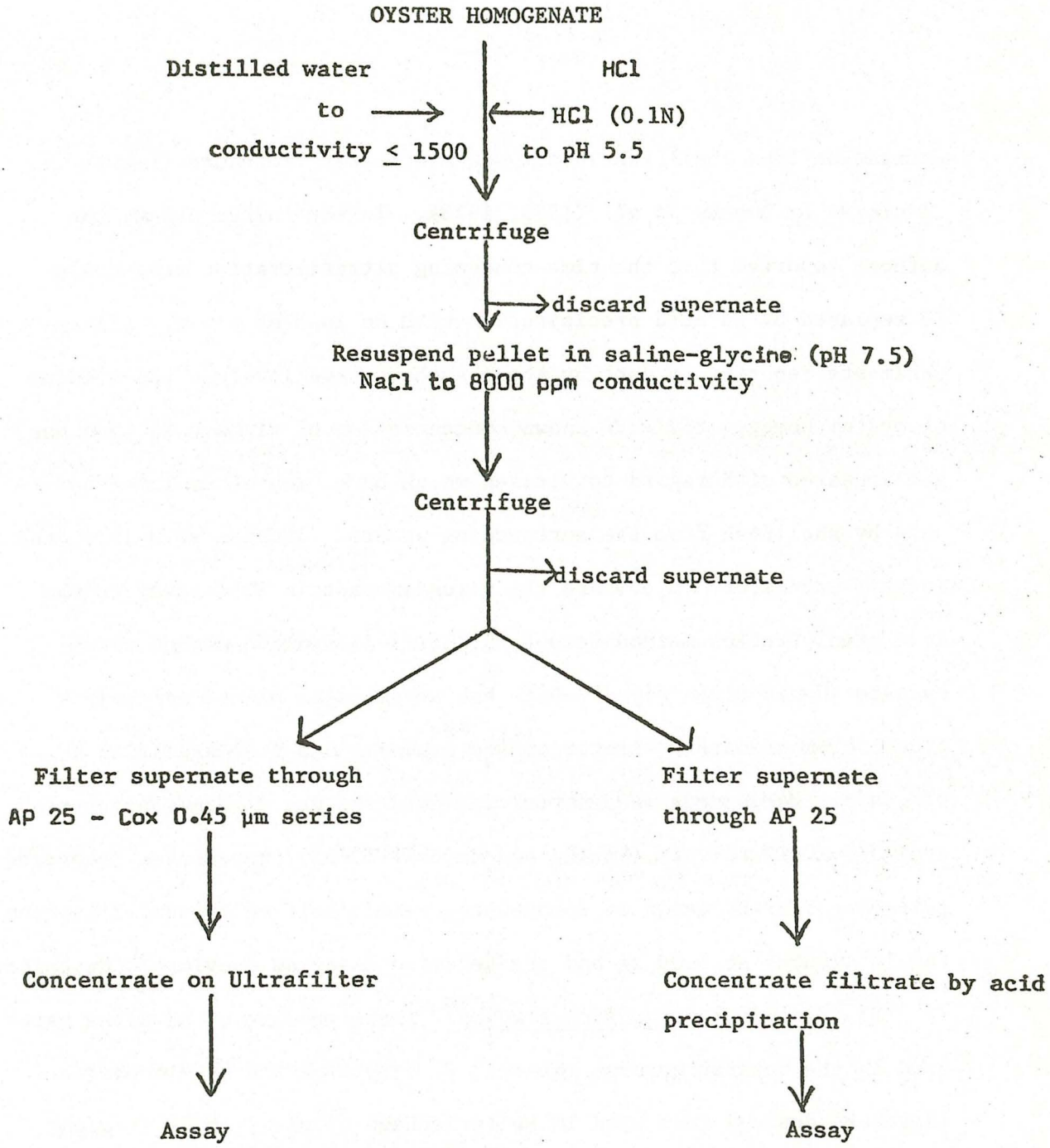


FIGURE 1

PROCEDURES FOR VIRUS CONCENTRATION FROM OYSTER

100-200 l of filtered seawater (15°C). Basins were then inoculated with poliovirus (approx. 1 x 10^4 pfu/ml) or MS-2 (approx. 1 x 10^5 pfu/ml). Shellfish were allowed to accumulate virus for a period of 18-24 hr at which time they were placed in the concrete raceways (12 x 1.2 x 1.5 m) which were supplied with running seawater. Samples were collected at intervals and examined for the presence of viruses. Temperature in the raceway was maintained at 15°C.

E) *Survival studies*. The ability of viruses to survive in seawater is obviously related to their likelihood of being taken up by shellfish. A series of survival studies were therefore undertaken. Human virus experiments involved the use of a non-vaccine strain of poliovirus type 3 (some authors have criticized the overuse of vaccine strains of polio in such experiments as they may not behave in a manner similar to their virulent counterparts). Viruses at a final concentration of 10^4 pfu/ml were inoculated into membrane diffusion chambers (Valley Machine and Engineering Co., Bel Grade, MT.) which contained 100 ml of either raw or 0.45 µm filtered seawater. (Note: Chambers contained a 0.45 µm - 15 nm filter series, this latter filter was used to keep viruses within the chamber). Chambers were then immersed in seawater which was maintained at 15°C throughout the experiment. Five ml samples were withdrawn at intervals for a period of 15 days and the surviving viruses enumerated as previously indicated.

Bacteriophage survival studies were carried out in dialysis bags

which contained 100 ml volumes of either raw or autoclaved seawater, or sterile saline (control). Each bag was inoculated with MS-2 phage at a final concentration of approximately 10^4 pfu/ml and immersed in seawater at 15°C. Samples were taken at 24-hour intervals and assayed for phage as previously described.

RESULTS AND DISCUSSION

A) *Comparison of methods for poliovirus extraction from shellfish*. As indicated elsewhere, data concerning the efficiency of the ultrafiltration method vs. the acid precipitation method for the extraction of human viruses from naturally infected oysters has not yet been published. Sobsey *et al.* (1978) had previously indicated that the two methods yielded similar efficiencies when using artificially seeded oyster homogenates. In our laboratory, acid precipitation was shown to be the superior method for virus extraction from naturally infected oysters. In two trials, the acid precipitation method yielded efficiencies of 84.6 and 36% respectively, while the time-consuming ultrafiltration method yielded 35.2 and 27% efficiencies (Table 9). While the data have established evidence for the comparative superiority of the acid precipitation method, the wide variation of efficiencies between these and other experiments not reported here indicate that this procedure does not yet represent the ultimate method for the extraction of viruses from shellfish. Efforts are continuing in this laboratory which hopefully will reveal a more consistently efficient method.

TABLE 9

Efficiency of Ultrafiltration and Acid Precipitation Methods in Extraction of Poliovirus type 1 from Naturally Infected Oysters

	Trial 1	Trial 2
Total virus pfu[a] before ultrafiltration step (UF)	2.3×10^5	2.0×10^5
Total virus in final concentrate	8.1×10^4	5.4×10^4
% efficiency of UF method	35.2%	27%
Total virus in homogenate before acid precipitation (AP)	1.3×10^5	2.0×10^5
Total virus in final concentrate	1.1×10^5	7.2×10^4
% efficiency of AP method	84.6%	36%

a = plaque forming units

B) *Survival in shellfish-growing waters*. All survival experiments were conducted at 15-18°C. The virulent strain of poliovirus type 3 was able to survive for a 10-day period in raw seawater, and for 13 days in 0.45 µm filtered seawater (Figure 2). The difference noted in survival rates suggested an active viricidal role for ambient seawater biota (<0.45 µm). This finding was consistent with those of other authors working in both fresh (Hermann *et al.*, 1974) and seawater (Mitchell and Jannasch, 1969; Akin *et al.*, 1974; Lo *et al.*, 1976) systems. The data also indicated that viruses would be able to sustain infectivity during passage through the entire aquaculture system as had been predicted in an earlier laboratory study using bacteriophage (Vaughn and Ryther, 1974).

Survival studies with MS-2 bacteriophage, which were carried out for a seven-day period, yielded survival profiles similar (in terms of % loss after 7 days) to those of poliovirus (Figure 3). This finding was of interest due to its potential impact on the use of rapid, inexpensive phage survival studies to predict the fate of human viruses (polio) in various aquatic systems.

C) *Shellfish depuration studies*. Numerous studies have been conducted to determine the relative viral depuration rates by shellfish. The inconsistencies in reported results suggest that depuration is likely to be dependent on species of shellfish, type of aquatic environment (*e.g.*, estuarine, coastal, polluted, unpolluted), and developmental

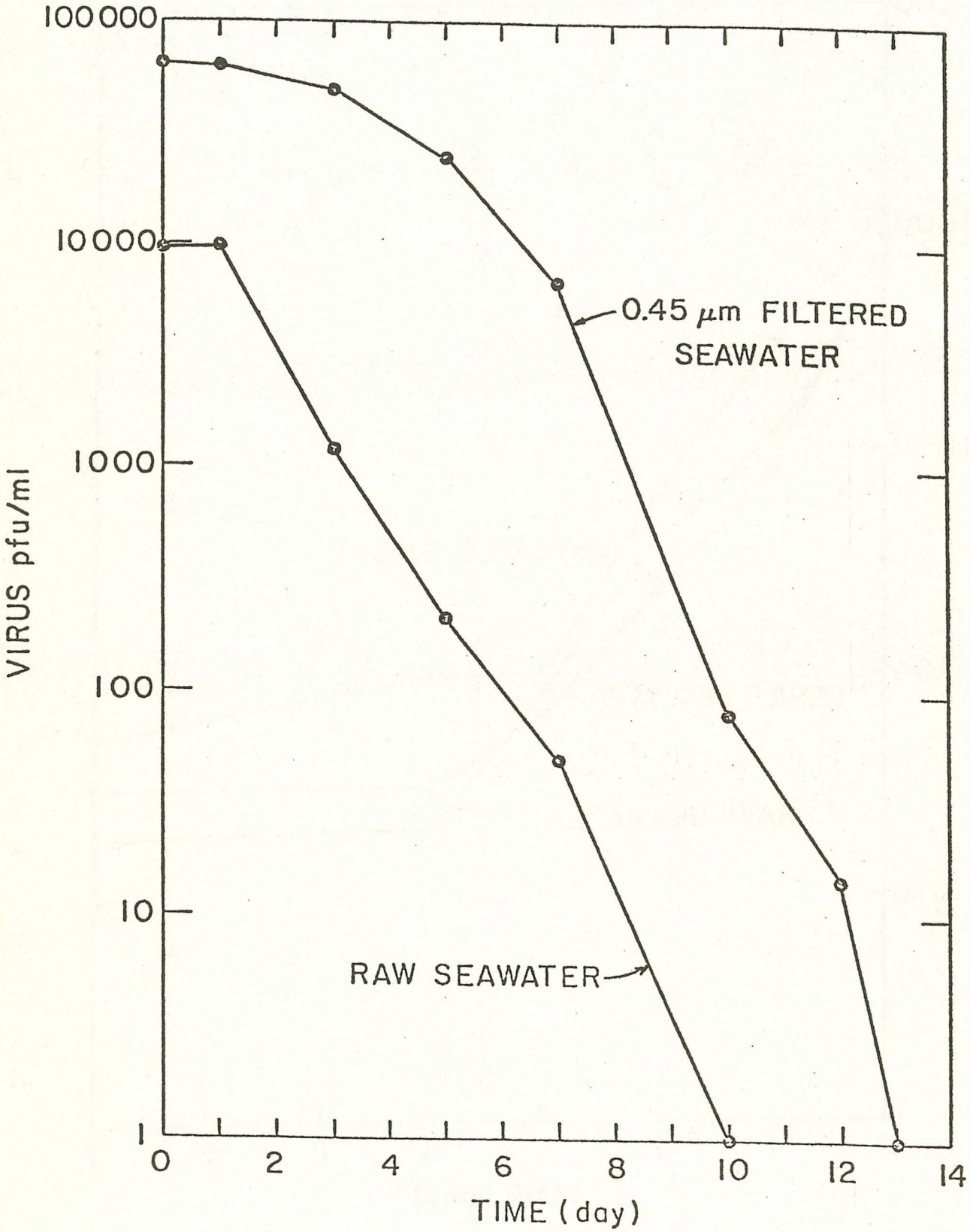


FIGURE 2

SURVIVAL OF A WILD TYPE POLIO TYPE 3 IN ESL RACEWAY WATERS

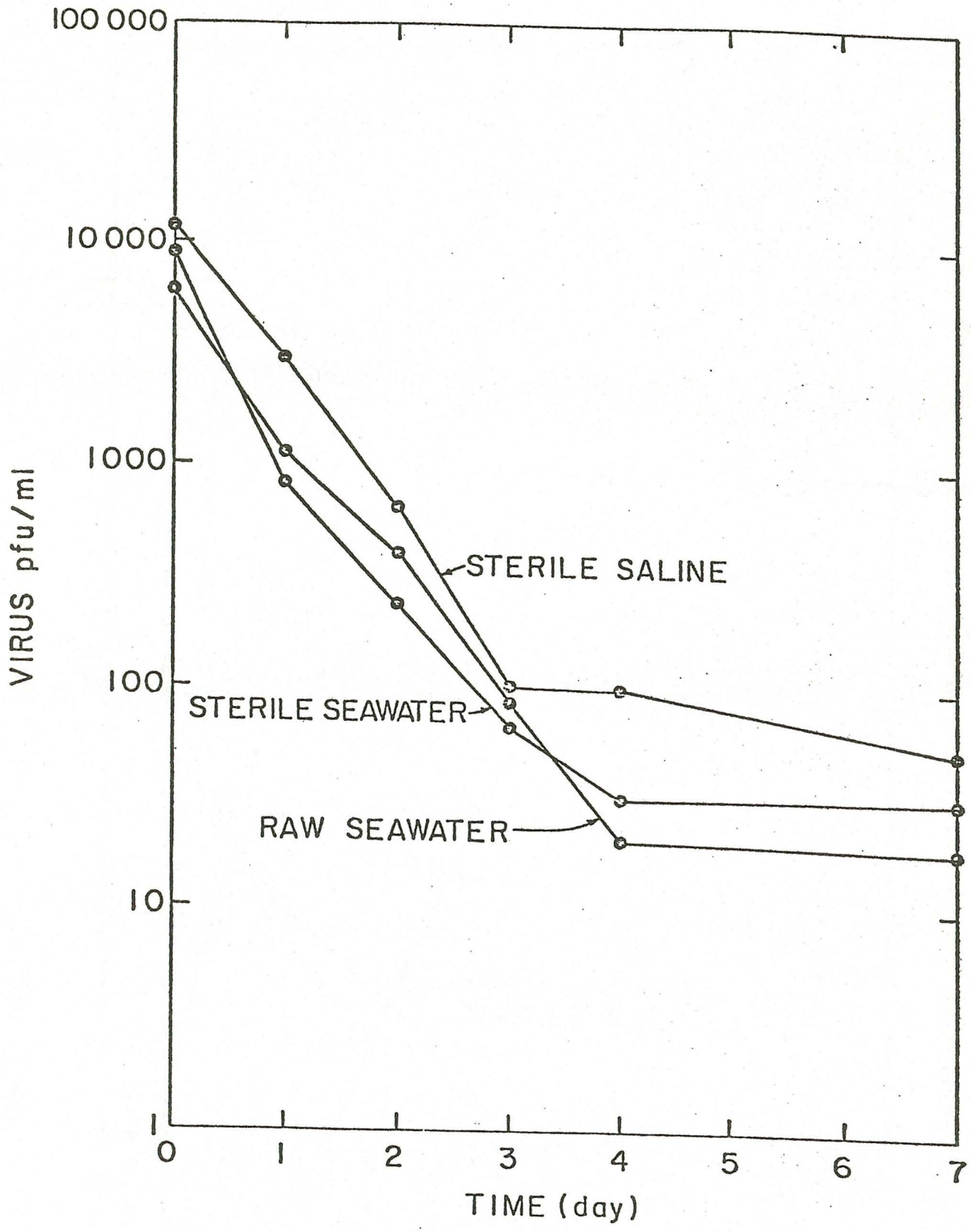


FIGURE 3

SURVIVAL OF MS-2 PHAGE IN ESL RACEWAY WATERS

state (*e.g.*, young and actively growing, mature adults) of the animals tested. Therefore, in order to accurately predict the likely depuration of particular animals growing in a particulate environment, virus studies should be carried out on representative animals in appropriate environments. The purpose of the studies discussed below was to specifically determine the time required for virus depuration in young animals residing in virus-free seawater.

In two trials, poliovirus was depurated from oysters to non-detectable levels in 7-17 days (Figure 4). These data were similar to those of several previous studies carried out in slightly different environments (Hedstrom and Lycke, 1964; Hoff and Becker, 1968; Vaughn and Metcalf, 1975). MS-2 depuration from oysters required more than 26 days (Figure 6). In an earlier laboratory study, Feng (1966) had indicated a similarly long depuration period for *Staphylococcus aureus* phage 80. Oysters demonstrated a greater uptake affinity for MS-2 phage than for poliovirus, with nearly 99% more phage accumulated per ml of homogenate. Analogous results had been previously reported for poliovirus and phage T4 uptake (Vaughn and Metcalf, 1975).

Elimination of MS-2 from quahogs (*M. mercenaria*) required 17 days (Figure 5). This retention time appeared to be somewhat longer than that predicted by Liu *et al.* (1967) for depuration of poliovirus types 1 and 3.

Based upon the above results, it would appear that using MS-2

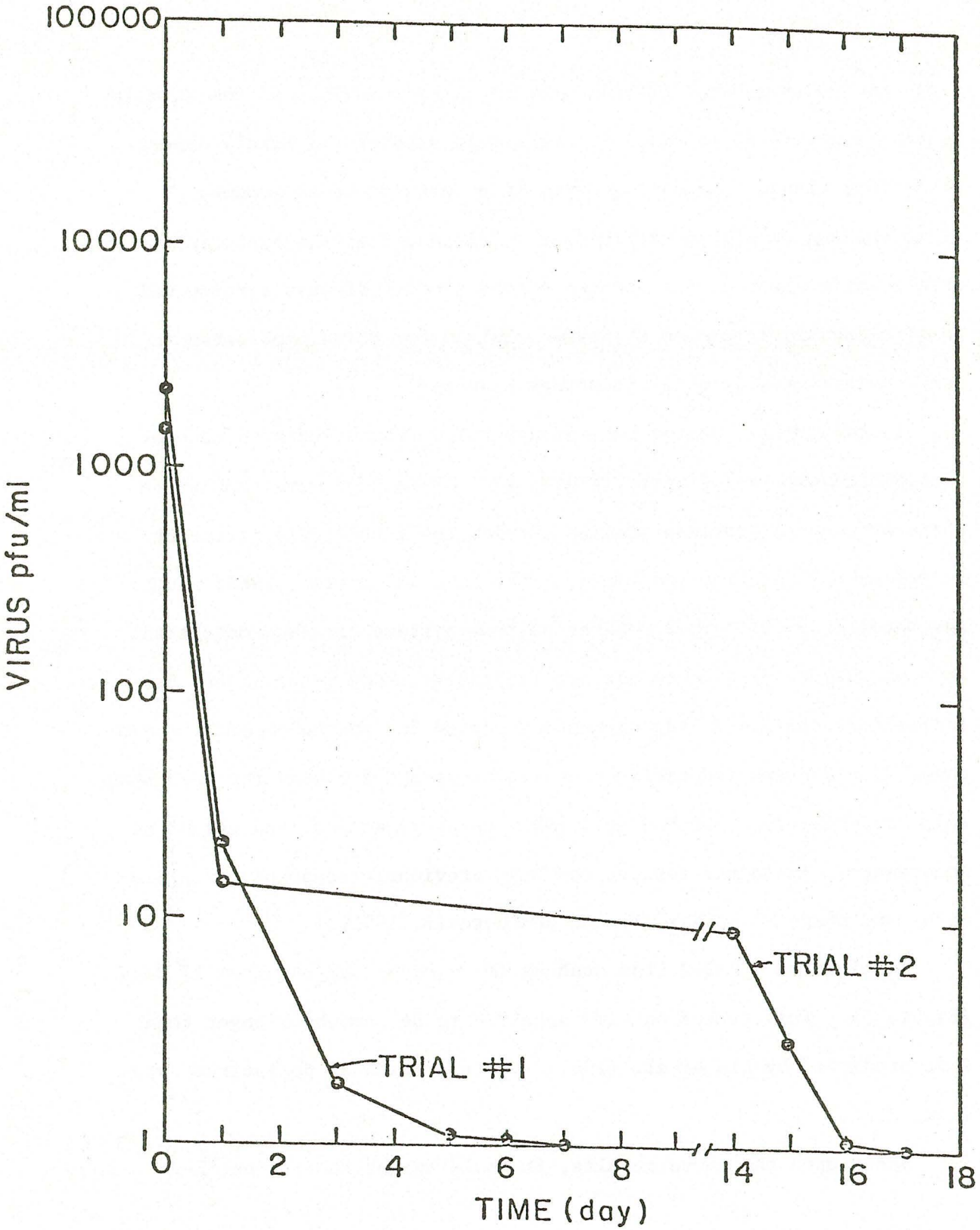


FIGURE 4

DEPURATION OF POLIOVIRUS TYPE 1 (LSC) BY CRASSOSTREA SP

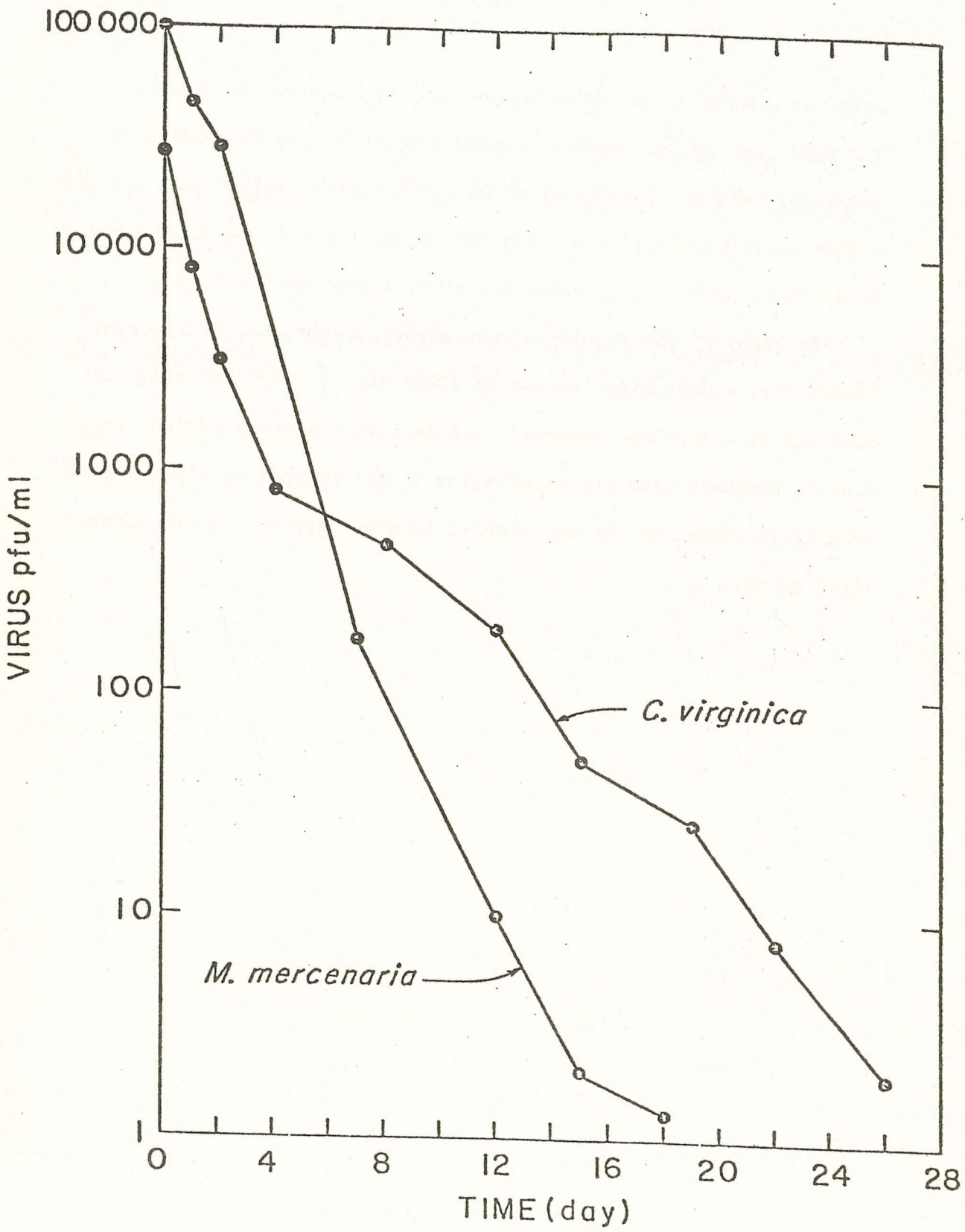


FIGURE 5

DEPURATION OF MS-2 PHAGE BY CRASSOSTREA and MERCENARIA SP

phage as a human virus model for determining depuration rates in a specific system would provide representative data with a built-in margin of safety. Caution is urged in overextrapolating such a model system as it may have little bearing on the behavior of virus types other than polio (*e.g.*, Coxsackie, ECHO, Adeno, Reo, etc.).

In light of the results of the above experiments, it is recommended that a depuration period of 20-25 days be used for shellfish residing in virus-free seawater. It is felt that this period would provide adequate time for elimination of all poliovirus types. This conclusion cannot as yet be extended to other members of the enterovirus group.

DISCUSSION AND FUTURE PROSPECTUS

The data of the present study indicate that the oysters cultured in a waste recycling aquaculture system under the regimes described herein consistently exhibit low, sewage effluent derived contaminant levels. This is perhaps, particularly interesting in that bivalve molluscs have often been selected as environmental pollution indicators due to their supposed ability to accumulate pollutants (Goldberg, 1975). It should, however, be noted that, unlike in natural systems, the bivalves in the present study were not cultured at a sediment-water interface. In natural seawater systems subjected to heavy metal pollution sediments often form an important sink for contaminants (see Chen, 1974; Rohatgi and Chen, 1975; Zirino and Yamamoto, 1972). Consequently the elevated levels of metal contaminants recorded in polluted environments may be more representative of the bivalve-sediment relationship than the bivalve-water column relationship.

The F.D.A. "alert" levels for trace metal contamination are derived from data collected in surveys of trace metal contaminants in selected shellfish species collected from both coasts of the United States. Data is compounded by species with emphasis being placed on *C. virginica* and *M. mercenaria* as these are major commercial products. Neither of these species were examined in the present study as both had been previously examined for growth potential and found to be of

little value in waste recycling systems (Mann and Ryther, 1976). Throughout the preceding text the assumption has been made that data derived for these East Coast species, *C. virginica*, could be comparable to those for *C. gigas* as their ecological niches are very similar.

The data of the present study are considerable and consistent in suggesting that public health hazards directly attributable to the trace metal and organic contaminants in oysters grown in a waste recycling-aquaculture system of the type described herein are minimal. However, this should not be taken to mean that in certain circumstances metal contamination would not be severe, or that other contaminant-related, biological or physical problems would not be encountered which would present major obstacles to the eventual on-site application of waste recycling technology. The present data also suggest that the Cranston sewage treatment plants are operating efficiently and producing effluent that is well within acceptable standards as defined by the EPA. Such a situation is undoubtedly not universal. The consistently low contaminant levels in both effluents and cultured organisms indicates the importance of maintaining high quality, low contaminant effluent for application in the present food chain.

Aside from public health related problems perhaps the other remaining question yet to be answered is that of the economic feasibility of waste recycling systems. Since its inception approximately seven

years ago the waste recycling aquaculture project at Woods Hole has accumulated a large number of data sets on various aspects of its operation. These include nutrient transformations in mass phytoplankton cultures and associated changes in primary productivity, factors controlling species competition in phytoplankton culture, the comparative growth of different species of bivalves and environmental factors affecting shellfish growth, and uptake and depuration of trace metal and organic contaminants and viruses (present study). At the time of writing a cooperative program with the Department of Resource Economics, U. Massachusetts, Amherst had recently been initiated to examine how the available biological and engineering data sets may be used to construct a model of a working waste recycling aquaculture facility, and subsequently evaluate its economic feasibility. This will be described in later reports.

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A study of 24 weeks duration was carried out in which oysters (*Crassostrea gigas*) were grown in four regimes. These were: (i) on phytoplankton cultured in a mixture of secondary treated sewage effluent and seawater for a period of 12 weeks followed by a second 12 week period of feeding on phytoplankton cultured in a "clean", inorganically enriched regime; (ii) as for (i) except that the secondary effluent was sand-filtered prior to use; (iii) as for (ii) except that the effluent was charcoal filtered prior to use; and (iv) using "clean", inorganically enriched phytoplankton food for the 24 week duration. At intervals of two weeks, populations of oysters were removed for assay for trace metals (Cd, Cr, Cu, Hg, Ni, Pb, Zn) and organic contaminants (hydrocarbons, P.C.B.'s). No significant accumulation or depuration of any metal or organic contaminant was evident in any of the regimes. In terms of these contaminants, all oysters are within acceptable edible standards as set by F.D.A.

A series of experiments was carried out to examine the public health implications of enterovirus survival in a mollusc culture system fertilized with secondary treated sewage effluent. Using MS-2 bacteriophage and vaccine strain poliovirus it would appear that depuration could be effected in 20-25 days in *C. gigas* at 15°C. However, this does not mean that such a time span would be adequate for other enteroviruses. Further work is required in this area.

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2. Oyster
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